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In Search of Topical Agricultural Biofungicides: Properties of the Recombinant Antimicrobial Peptide TrxAq-AMP Obtained from *Amaranthus quitensis*

Diego Alem[1], Paola Díaz-Dellavalle[2], Carolina Leoni[3], Salvatore G De-Simone[4,5], Agustín Correa[6], Pablo Oppezzo[6] and Marco Dalla Rizza[1]*

[1]*Laboratorio de Proteínas, Unidad de Biotecnología, Estación Experimental Wilson Ferreira Aldunate, Instituto Nacional de Investigación Agropecuaria (INIA Las Brujas), Canelones, Uruguay*
[2]*Departamento de Ciencia y Tecnología de los Alimentos, Facultad de Ingeniería y Tecnologías, Universidad Católica del Uruguay (UCU), Montevideo, Uruguay*
[3]*Sección Protección Vegetal, Programa Nacional de Investigación de Producción y Sustentabilidad Ambiental, Estación Experimental Wilson Ferreira Aldunate, Instituto Nacional de Investigación Agropecuaria (INIA Las Brujas), Canelones, Uruguay*
[4]*Center for Technological Development in Health (CDTS)/National Institute of Science and Technology for Innovation in Neglected Diseases (INCT-IDN), FIOCRUZ, Rio de Janeiro, RJ, Brazil*
[5]*Biochemistryof Proteins and Peptides Laboratory, Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro, RJ, Brazil.*
[6]*Unidad de Proteínas Recombinantes – Institut Pasteur de Montevideo, Uruguay*

Abstract

Synthetic pesticides have a positive impact on food production. However, there are concerns due to the outbreak of resistance along with negative side effects on human health and the environment. New active compounds and control strategies are needed for the management of phytopathogens. Antimicrobial peptides (AMPs) are evolutionarily conserved components of the innate immune response in almost all organisms that constitute an interesting source of potential molecules for use as pesticides. The naturally derived antimicrobial peptide Aq-AMP (*Amaranthus quitensis*-Antimicrobial Peptide), obtained from *Amaranthus quitensis*, is cysteine-rich with activity against several phytopathogens. In the present work, we report on the expression in *Escherichia coli* of functionally active Aq-AMP fused to thioredoxin (TrxAq-AMP). The *in vitro* antifungal activity of purified TrxAq-AMP was confirmed against *Alternaria solani*, *Fusarium oxysporum* f. sp. *lycopersici*, *Pencillium digitatum* and *P. italicum*, as well as the *in vivo* control of *P. digitatum* in oranges. We demonstrated the stability of TrxAq-AMP in a range of pH (from 3 to 11) and at temperatures from 0°C to 100°C. Furthermore, it maintained activity after digestion with various proteases and it displayed no haemolytic activity suggesting a highly stable and safe molecule. For topical application we present AMP with no haemolytic/phytotoxic activity that is effective, stable to a wide range of temperature and pH, and resistant to protease activity. Besides, this molecule is naturally stored in the seed, easy to extract and potentially produced through molecular farming. These findings encourage further biotechnological research on topical application of AMPs, especially in relation to molecule bioavailability.

Keywords: Antimicrobial peptides; *Alternaria solani*; *Fusarium oxysporum* f. sp. *lycopersici*; *Penicillium* sp.; Heterologous expression

Abbreviations: AMPs: antimicrobial peptides; Ac-AMP: *Amaranthus caudatus*-Antimicrobial Peptide; Aq-AMP: *Amaranthus quitensis*-antimicrobial peptide; LB: Luria Bertani; Fmoc: Fluoren-9-Methyloxycarbonyl; HPLC: High Performance Liquid Chromatography; IMAC: Immobilized Metal Affinity Chromatography; IPTG: Isopropyl B-D-1-Thiogalactopyranoside; MALDI: Matrix-Associated Laser Desorption Ionization; MFC: Minimal Fungicidal Concentration; MIC: Minimal Inhibitory Concentration; PCR: Polymerase Chain Reaction; PDA: Potato Dextrose Agar; PDB: Potato Dextrose Broth; ON: Overnight; SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; Trx: Thioredoxin

Introduction

Synthetic pesticides have a significant impact on food production by reducing crop losses due to pests, disease and weeds. Yet, these positive benefits are being reconsidered in light of negative side effects. Instances of resistance along with impacts on human health and the environment are driving the search for new active compounds and control strategies for the management of plant pathogens. Searches are focused on new molecules with various properties including high specificity for target pathogens, effectiveness at low dosages and biodegradability, with a low risk for the development of resistance by the pathogen population. A highly promising group of molecules are antimicrobial peptides (AMPs), which are evolutionarily conserved components of the innate immune response in almost all organisms and constitute a potentially interesting source of molecules for use as pesticides [1-4]. Most plant AMPs are small molecules with less than 50 amino acids, a molecular weight below 10 kDa, net positive charge, amphipathic and cysteine-rich with disulfide bonds that confer high stability [2,5].

Several AMPs have been reported for controlling plant pathogens [2,5-9]. Within the Amaranthaceae family, Broekaert et al. [10] described two molecules with 29 and 30 amino acids (Ac-AMP1 and Ac-AMP2) with *in vitro* activity against several fungi, most of them being phytopathogens (*Alternaria brassicola*, *Ascochyta pisi*, *Botrytis cinerea*, *Colletotrichum lindemuthianum*, *Fusarium culmorum*, *Trichoderma hamatum*, *Verticillium dahliae*) and high stability at different pH, temperatures and protease digestion. More recently,

***Corresponding author:** Marco Dalla Rizza, Laboratorio de Proteínas, Unidad de Biotecnología, Estación Experimental Wilson Ferreira Aldunate, Instituto Nacional de Investigación Agropecuaria (INIA Las Brujas), Canelones, Uruguay, Tel: +598 3677641; Fax: 598 3677609; E-mail: mdallarizza@inia.org.uy

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AMPs from, *Amaranthus retroflexus* and *A. hypochondriacus* have been reported [11,12]. Also, our group identified and purified two peptides from *A. quitensis* seeds (Aq-AMP1 and Aq-AMP2) with the same sequence as the peptides isolated from *A. caudatus* (Ac-AMP1 and Ac-AMP2, respectively) [13].

Most analyses of AMP activity have been performed *in vitro*. However, a laboratory setting neglects the importance of the environment on the interactions between the plant, pathogen and AMP that can affect the *in vivo* activity. The high production costs associated with AMPs can explain the current limitation in analysis. Advances in biotechnological procedures could overcome these constraints by heterologous production using bacteria and yeasts [14-18] or transgenic plants as biofactories [5,19-22]. Improvements in production would be a breakthrough for not only research purposes, but also for the application of AMP to plant protection. Several peptides already show satisfactory efficacy against many agricultural pathogens, .with strong potential for the production of a commercial fungicide. These AMPs must meet certain requirements, .with strong potential for the production. They must be effective and easy to produce [8]. Besides their biological properties, AMPs are easily identified molecules, allowing traceability and facilitating the regulatory requirements for registration of biopesticides for agricultural use.

In the present work, our objective was to express a functional form of the naturally derived antimicrobial peptide obtained from *Amaranthus quitensis* seeds (Aq-AMP), fused to thioredoxin in *Escherichia coli*. We further demonstrated the functionality of its *in vitro* antifungal activity through assays against four filamentous fungi of agricultural relevance. Finally, the *in vivo* activity was tested against *Penicillium digitatum* in Valencia oranges.

Materials and Methods

Materials

Ni-NTA affinity chromatography was used for the purification of the fusion protein. The endonucleases, Taq DNA polymerase, T4 ligase and DNA encoding a His-tag and the S-Tag were from Novagen (Madison, WI, USA). The Vydac reverse-phase C18 column was from Altech Associates, Inc. (Dearfield, IL, USA) and His Trap HP columns from GE-Healthcare (Pittsburg, PA, USA). Protein molecular weight markers, imidazole, lysozyme, protease inhibitors, IPTG, sequence reagents and synthesis solvents were purchased from Sigma-Aldrich Co (St Louis, Mo, USA). Bacto tryptone and Bacto yeast extract were from Difco-Becton Dickinson Co (Franklin Lakes, NJ, USA). Fungaflor 75 PS was acquired from Janssen Pharmaceutica (Titusville, NJ, USA) while Potato Dextrose Broth (PDB) and Potato Dextrose Agar (PDA) were from Hi Media (Mumbai, India).

Strains and expression vector

The *Escherichia coli* strain DH5α was used for plasmid propagation of the expression clone pET 32a. The *E. coli* strains BL21-DE3 pLys (Novagen) and Shuffle (New England Biolabs) were used to evaluate the optimal expression conditions of the fused protein. The expression plasmid pET32a$^{(+)}$ (Novagen) was used to express the recombinant construct, which was fused to thioredoxin (Trx) (109 aa) at the N-terminal extreme, followed by a 6x His-Tag, a thrombin cleavage site and the Aq-AMP (86 aa) protein in the C-terminal region (Trx/His/Aq-AMP).

Microorganisms

Four fungal strains of agricultural relevance were used for the *in vitro* and *in vivo* experiments. *Alternaria solani* (isolated from diseased tomato leaves), *Fusarium oxysporum* f.sp. *lycopersici* (isolated from diseased tomato stems), *Penicillium digitatum* and *P. italicum* (both isolated from orange fruits), were supplied by the Plant Pathology Laboratory of the National Institute of Agricultural Research of Uruguay. All fungal strains were cultured on PDA at 24°C for 7 days. Spore suspensions were obtained in a sterile solution of 0.1% Tween-20 by filtration through gauze and centrifugation (7,000×g; 20 min). Stock spore suspensions were adjusted to 2×10^6 spores/ml after counting in a cell counting chamber and stored in 20% glycerol at -80°C until use.

Peptide synthesis

The peptide Pses3 (WRQRYYRYVHGYMGPKGYTR) from *Sesamum indicum* was synthesized by the solid-phase synthesis method in a PSS-8 Pep Synthesizer (Shimadzu, Kyoto, Japan) according to the fluoren-9-methyloxycarbonyl (Fmoc)-polyamide active ester chemistry [9]. The synthesized peptide was purified using a reverse-phase C_{18} column and the purity was confirmed by matrix-associated laser desorption ionization (MALDI) mass spectroscopy (Kratos Kompact MALDI).

Cloning of the complete gene protein Aq-AMP2 in pET32a$^{(+)}$

A. quitensis seeds were collected at the National Institute of Agricultural Research of Uruguay (34°40'19" S, 56°20'24" W), accessions numbers UB_Aq14.1 and UB_Aq14.2 deposited at the MVFA herbarium, Agronomy Faculty, Universidad de la República, Uruguay.

DNA template was extracted from seeds with specific primers (Aq-AMP2ForwNco1:AAACCATGGTGAACATGAAGAGTGTTGC;Aq-AMP2 Rev_Xho1: TTTCTCGAGTCATGGACTACCAGCACCAG) designed on the basis of *A. caudatus* sequence information [23] and the complete protein was amplified by polymerase chain reaction (PCR) using Phusion polymerase (Finnzymes). A band with the expected molecular weight was obtained and purified by agarose gel (QIA Quick Gel Extraction kit, Qiagen). The purified PCR product and the pET32a$^{(+)}$ vector were digested with the restriction enzymes NcoI and XhoI (Fermentas) according to the manufacturer's recommendations. After digestion both products were ligated with the Quick Ligation Kit (New England Biolabs) and transformed in XL1-Blue electrocompetent cells. Positive colonies were selected by digestion of purified plasmids with the restriction enzymes mentioned above and confirmed by DNA sequencing.

Expression test of the construct Trx/His/Aq-AMP

Expression assays were performed at two different temperatures (16°C and 37°C) and in two different expression strains, BL21(D3)pLys and Shuffle T7 Express lysY (New England Biolabs). For the expression screening, 200 ml LB media (Luria-Bertani) complemented with 100 µg/ml ampicillin were inoculated with 2 ml overnight (ON) culture, and grown at 37°C.

After reaching a OD_{600nm}=0.8, cultures were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), for 4 h at 37°C or ON at 16°C. After induction, cells were recovered by centrifugation and resuspended in buffer A (50 mM $NaPO_4$ pH 8.0, 0.5 M NaCl, 20 mM imidazole) with lysozyme (1 mg/ml) (Sigma Aldrich), protease inhibitors EDTA-Free (Roche Inc. USA), and frozen at -80°C.

Cells were thawed and sonicated with a micro-tip, performing 3 pulses of 20 seconds (2 seconds with a 1 second of rest) 35% amplitude and centrifuged at 17000×g for 40 min at 4°C. Soluble fractions were filtered on a 0.45 µM membrane and injected on a column of His Trap HP 1 ml (GE-Healthcare), previously equilibrated in buffer A. Subsequently the column was washed with 10 volumes of buffer A and eluted with buffer B (50 mM $NaPO_4$ pH 8.0, 0.5 M NaCl, 0.5 M imidazole). The eluted fractions were analyzed by SDS-PAGE.

Tricine SDS-PAGE

A solution of solubilizing buffer (SSB), composed of 0.5 M Tris (pH 6.8), 20% glycerol (v/v), 10% SDS (w/v), 10% β-mercaptoethanol (v/v), and 0.1% bromophenol blue (w/v), was freshly prepared. The solution of peptides was mixed with the fresh SSB in a ratio of 2:1, and the mixtures were incubated at 95°C for 5min. The samples were run on a discontinuous Tricine-SDS-PAGE with a 10% stacking gel and a 12% separating gel [24].

Expression and characterization of the fusion protein Trx/His/Aq-AMP

Expression in 1 l of LB media was carried out in the Shuffle strain. Induction was performed with 1 mM IPTG at 16°C ON and cells were recovered by centrifugation. The pellet was resuspended as previously described. Recombinant protein was purified by immobilized metal affinity chromatography (IMAC) using a 1 ml HisTrap column (GE Healthcare) and eluted in a continuous gradient (0-100% of buffer B). Purified protein was then injected in a Superdex 10/300 (GE Healthcare) equilibrated in buffer A for the determination of the oligomeric state.

In vitro antifungal activity assay: MIC and MFC

The *in vitro* antifungal activity was determined by a quantitative micro-spectrophotometric assay [25]. Inhibition of growth was measured in 96-well microtiter plates at 595 nm (Multiscan FC, Thermoscientific, Shangai, China). In each well, 50 µl of 50% PDB with 2×10^4 spores/ml of fungi and 50 µl of a peptide suspension at the desired concentration were mixed. After 30 min at 24°C to allow sedimentation, absorbance was measured (Abs0), then the plates were incubated at 24°C for 48 h and absorbance was measured again (Abs48). Water was used as the negative control (AbsW), thioredoxin at 128.0 µM was tested to rule out any effect on fungal growth, and the peptide concentrations used were 0.0, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, 64.0 and 128.0 µM. The minimal inhibitory concentration (MIC) of the peptides was defined as the lowest concentration of peptide that inhibited growth by more than 90% after 48 h as compared to the control. Each fungal assay, by treatment and concentration, was performed in triplicate. MIC was calculated as the average of the three replications.

The *in vitro* minimal fungicidal concentration (MFC) was determined as described [26]. After 48 h of incubation, 20 µl was subcultured from each well that showed no visible growth (growth inhibition of over 98%), from the last positive well (growth similar to that for the growth control well), and from the growth control (extract-free medium) onto PDA plates. The plates were incubated at 24°C until growth was seen in the growth control subculture. The minimum fungicidal concentration was regarded as the lowest peptide concentration that did not yield any fungal growth on the solid medium. For each fungal isolate, treatment and concentration was tested in triplicate. MFC was calculated as the average of the three replications.

In vivo antifungal activity assay

The *in vivo* activity was assayed in fresh Valencia orange fruits (*Citrus sinensis* L. Osbeck), harvested two days before the experiment began. Fruits were surface disinfested with 200 ppm NaOCl solution for five minutes, rinsed three times in distilled water and then air dried on the bench. Next, fruits were injured with a sterile needle (3 mm depth, 4 lesions per fruit along the equator) and inoculated with 5 µl of an aqueous mixed suspension of the pathogen *P. digitatum* (2×10^5 conidia/ml) and the peptide at the desired concentration (64, 32 or 6.4 µM). As negative and positive controls, water and Imazalil (1000 ppm of the active ingredient of the commercial fungicide Fungaflor 75 PS, Janssen Pharmaceutica) were used, respectively. Thioredoxin was not included as a third control since no antifungal activity was found in the *in vitro* experiment described above. After inoculation, fruits were kept at 20°C and 90% relative humidity for 7 days before evaluation. Fruits were scored according to the presence/absence of lesions. The probability of occurrence of healthy fruit was analyzed with a Binomial distribution and Logit link function, employing the GENMOD procedure of SAS Statistical Package (Statistical Analysis System, Version 9.2, 2008).

Peptide stability

All peptide stability assays were performed with the *F. oxysporum* f.sp. *lycopersici* strain, and the activity of the peptide was evaluated by the quantitative micro-spectrophotometric technique described above and using a peptide concentration of 4.0 µM, the MIC established in the *in vitro* antifungal activity assay. There was considered to be no activity when the absorbance was similar to the AbsW (with water and no peptide).

pH stability: The peptides were incubated at 37°C for 1 h in different buffer solutions, and then adjusted to pH7 before testing for antifungal activity. The solutions used at 100 mM were: glycine/HCl (pH 3.0), acetate/acetic acid (pH 5.), monobasic/dibasic sodium phosphate buffer (pH 7.0), Tris/HCl (pH 9.0), and monobasic/dibasic sodium phosphate (pH 11.0).

Thermostability: Peptides were dissolved in PBS (pH 7.4) and incubated for 1 h at 0, 60, 80 and 100°C. The solutions were then cooled to 4°C and centrifuged (15000×g; 10 min) to remove any precipitation.

Protease digestion: The peptides were digested either with 1 mg/ml of pronase E, proteinase K or trypsin. The reaction was performed at 37°C for the first two proteases and at 25°C for trypsin. The reaction was stopped by incubating first at 95°C for 5 min and then the mixture was cooled to 4°C.

Haemolytic activity

Haemolytic activity for the two peptides (TrxAq-AMP and Pses3) was determined at different concentrations (0.0, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, 64.0 and 128.0 µM) as described [27]. Briefly, 3 mL of fresh sheep blood cells were washed three times with PBS (pH 7) then suspended in PBS (10% v/v). Blood cell suspension (50 µL) was added to a peptide PBS suspension (50 µl), mixed gently, incubated at 37°C for one hour and centrifuged (2000×g; 5 min). The supernatant was transferred to a 96-well microtiter plate and the absorbance of the sample (AbsS) measured at 450 nm (Multiscan FC, Thermoscientific, Shangai, China). As positive and negative controls, Triton 0.2% X-100 (100% hemolysis=AbsT) and PBS (0% hemolysis=AbsW) were used. Haemolysis % (H%) was determined as H%=[(AbsS – AbsW) / (AbsT – AbsW)]x100.

Results

Evaluation of different expression conditions and large scale purification of the recombinant protein Trx/His/Aq-AMP

In order to obtain greater amounts of the recombinant protein Trx/His/Aq-AMP we evaluated different expression conditions including two *E. coli* strains (BL21(D3) pLys and Shuffle) and two different induction temperatures (16°C ON and 37°C for 4 h). The results showed better expression in the Shuffle strain with an induction temperature of 16°C ON (data not shown). Next, we used these conditions for a large scale production and purification (1 l) of Trx/His/Aq-AMP. Our results showed that the recombinant protein could be obtained at a final yield of 38 mg/l (Figure 1A and 1B). Following this we injected the recombinant protein into a Superdex 200 10/300 to determine its oligomeric state. The result indicated a molecular mass of around 500 kDa, which corresponds to 20 Trx/His/Aq-AMP monomers (Figure 1C). This result was confirmed by dynamic light scattering (data not shown).

In vitro fungal assays

Both the synthetic and bacterially-expressed peptides presented antifungal activity against the four phytopathogens assayed. The thioredoxin alone did not display activity at the highest concentration of 128.0 µM (Table 1). The MIC and MFC values against the four fungi for TrxAq-AMP were 4.0 µM and 8.0 µM, respectively. Pses3 presented a different response to each pathogen, with MIC values of 4.0 µM for *A. solani* and *F. oxysporum* f. sp. *lycopersici* and 2.0 µM for *Penicillium* sp., and MFC values of 8.0 µM for *A. solani* and 4.0 µM for the other three pathogens.

In vivo fungal assay

In the *in vivo* assay, only the peptide TrxAq-AMP at 64.0 µM showed antifungal activity against *P. digitatum* controlling 62% of the lesions, which was lower than the positive control, the fungicide Imazalil (84%; $p<0.068$). The peptide Pses3 at 64.0 µM only controlled 6% of the infection lesions, similar to the 3% observed for the negative control, water treatment ($p<0.001$) (Table 2 and Figure 2). No phytotoxic activity was observed at any dose of the assayed peptides.

Peptide stability and haemolytic activity

Stability differed between the synthetic peptide, Pses3, and the recombinant peptide TrxAq-AMP. TrxAq-AMP maintained its activity over a wide range of pH (from 3 to 11), temperatures (0, 60, 80, 100°C) and even after exposure to proteases for 1h and 16 h. The

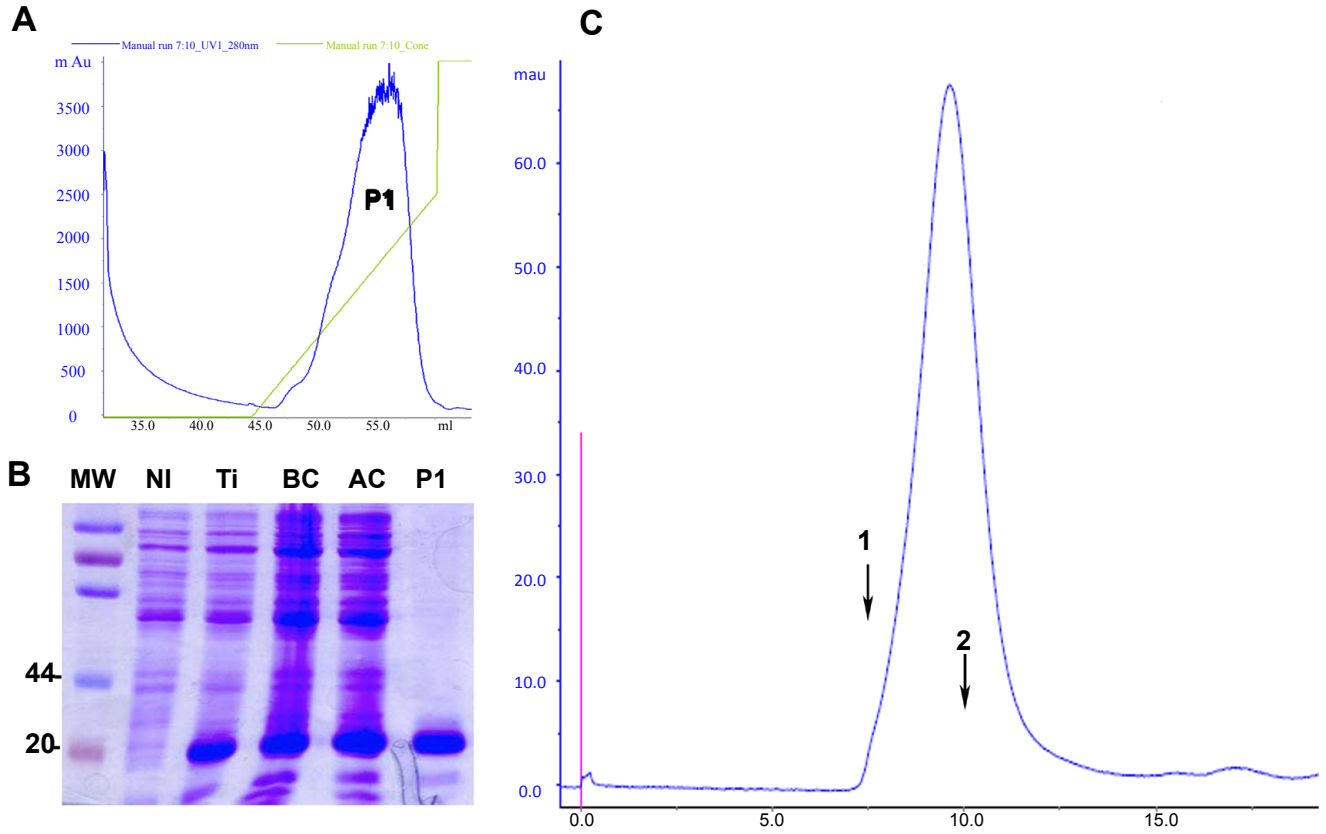

Figure 1: Purification and characterization of Trx/His/Aq-AMP. A. Chromatogram obtained from IMAC purification of Trx/His/Aq-AMP. B. SDS/PAGE 12% of the purified protein. C. Size exclusion chromatography in a Superdex 200 10/300 of the P1 fraction. P1, eluted peak from IMAC purification. MW, molecular weight (kDa), NI, not induced; TI, total induced; BC, before column; AC; after column. Arrow 1 corresponds to the retention volume for Blue Dextran (>700 kDa); arrow 2 corresponds to the retention volume for Ferritin (440 kDa).

	A. solani		*F. oxysporum f.sp. lycopersici*		*P. digitatum*		*P. Italicum*	
	MIC[a] (µM)	MFC[a] (µM)	MIC (µM)	MFC (µM)	MIC (µM)	MFC (µM)	MIC (µM)	MFC (µM)
AqTrxAMP	4	8	4	8	4	8	4	8
Pses3	4	8	4	4	2	4	2	4
Thioredoxin	ND [b]	ND	>128	ND	>128	ND	ND	ND

[a]MIC and MFC are the average of three replicates.

[b]ND=not determined.

Table 1: Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of the peptides Pses3 and TrxAq-AMP for the phytopathogenic fungi *Alternaria solani*, *Fusarium oxysporum* f.sp. *lycopersici*, *Penicillium digitatum* y *P. italicum*.

Treatment[a]	Estimated mean (%)[b]	Standard Error	Degrees of Freedom	Pr>Chi-Sq	
TrxAqAMP-6.4 µM	0.095	0.27	1	<.0001	a[c]
TrxAqAMP-32 µM	0.177	0.339	1	<.0001	a
TrxAqAMP-64 µM	0.621	0.27	1	0.068	b
Pses3-6.4 µM	0	67881.74	1	0.999	---
Pses3-32 µM	0.031	0.723	1	<.0001	a
Pses3-64 µM	0.063	0.523	1	<.0001	a
Control-Water	0.031	0.723	1	<.0001	a
Control-Imazalil 1000 ppm	0.839	0.351	1	<.0001	c

[a]Valencia orange fruits were co-inoculated with a conidial suspension of *P. digitatum* ($2x10^5$ conidia/ml) and either the peptide, the fungicide Imazalil or water.

[b]Means were estimated by the probability of occurrence of healthy fruits, analyzed with a Binomial distribution and Logit link function, employing the GENMOD procedure of SAS Statistical Package.

[c]Treatments statistically different are indicated with different letters.

Table 2: Estimated means for the percentage probability of occurrence of healthy fruits after co-inoculation with *P. digitatum* and the peptides TrxAq-AMP and Pses3.

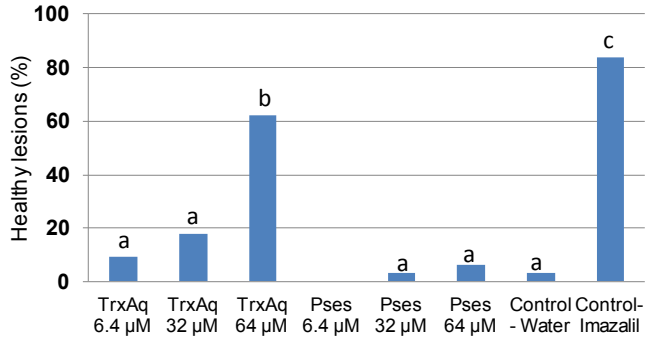

Figure 2: *In vivo* fungicidal activity of the peptides TrxAq-AMP and Pses3. The peptides were tested against *Penicillium digitatum* by evaluation of lesions formed on fresh Valencia oranges. Fruits were injured, inoculated with a mixed suspension of conidia (2×10^5 conidia/ml) with peptide at various concentrations and incubated at 20°C for 7 days at 90% relative humidity.

synthetic peptide Pses3 was sensitive to pH, temperature and proteases. As expected, antifungal activity of both peptides was reduced in the presence of $CaCl_2$ and KCl at 1.0 mM, and for TrxAq-AMP the activity was completely lost with $CaCl_2$ 5.0 mM.

The synthetic peptide Pses3 showed haemolytic activity with sheep blood cells at 32.0 µM, whereas TrxAq-AMP did not display any activity through to the highest concentration evaluated (128.0 µM).

Discussion

Prokaryotic expression of cysteine-rich plant AMPs frequently presents difficulties, mainly due to the presence of disulfide bridges, ease of degradation and toxicity to the host. In this work, a naturally derived recombinant peptide was shown to have *in vivo* functional characteristics similar to those reported for another expression platform [28]. This work advances studies on AMP by evaluating both *in vitro* and *in vivo* activity for the same peptide generated by different means. One was synthesized and the other expressed in bacterial cells. While both peptides showed strong activity *in vitro*, only the naturally derived TrxAq-AMP had antifungal activity *in vivo* against *P. digitatum*. The best explanation is that stability was conferred by the presence of three disulfide bridges generated during expression in *E. coli*, which allows the peptide to overcome pathogen reaction and adverse environmental conditions [5]. AMPs are not always tested for both *in vitro* and *in vivo* activity, in spite of the relevance of *in vivo* effects for selecting promising AMP molecules for further development as new potential fungicides.

The lack of *in vivo* experiments can be attributed to the high cost of "classical technology" for large scale AMP production, either by synthetic procedures or through prokaryotic factory cells. Nevertheless more recent reports demonstrate the feasibility of obtaining functionally active peptides and proteins on a large scale by using plants as bioreactors [29-31]. There are several expression platforms of recombinant proteins in plants. When the performance of human serum albumin (HSA) obtained using different plant tissues is compared, production from seeds is the most convenient in terms of cost effectiveness [31,32].

The *in vitro* and *in vivo* activity suggests a high potential for TrxAq-AMP as a bio-fungicide, as reported elsewhere for other peptide-pathosystems [18]. Our peptide presented a dose-dependent response, where MIC and MFC could be established and therefore the dose for *in vivo* experiments could be defined. With an aqueous solution of TrxAq-AMP at 64 µM, we obtained 62% probability of healthy fruit against *P. digitatum* in fresh Valencia orange fruits, compared with 84% probability of healthy fruit with the commercial fungicide imazalil. Additionally, TrxAq-AMP was highly stable at different pH values's and temperatures, in a broader range than the conditions assayed with Ac-AMP2 [10]. In addition, TrxAq-AMP showed no haemolytic activity suggesting a high degree of safety. This last property of TrxAq-AMP is a significant advantage compared to the linear peptide Pses3, which was highly haemolytic suggesting a potential health risk. These results indicate TrxAq-AMP may be a promising alternative to conventional fungicides. Conventional fungicides give rise to concern due to the development of fungal resistance and their negative side effects on human health and the environment. In particular, the citrus industry is threatened by difficulties in controlling post-harvest decay [4].

The sequence of the molecule from *A. quitensis* seeds (Aq-AMP2) is identical to that reported for *A. caudatus* (Ac-AMP2) [10,13]. After demonstrating the potential of TrxAq-AMP as a novel class of bio-fungicides, the challenge is to go further in scaling up expression for mass production. Based on actual knowledge, the most promising path is the use of eukaryotic platforms. With these platforms, both technological adjustments and economic trade-offs should be considered.

Topical application of naturally derived AMPs for controlling pathogens is under development. Our group is focusing on phytopathogens of agricultural relevance, mainly postharvest pathogens and pathogens affecting fruits for fresh consumption. To be considered as a potential biopesticide, AMPs should combine certain intrinsic properties such as no haemolytic activity, and stability after exposure to proteases as well as to a range of pH and temperatures.

Also, other desired properties are high specificity for target pathogens, effective at low dosages, biodegradability and low risk for resistance development by the pathogen population.

Our next step is to scale-up Aq-AMP production, and for this we are evaluating prokaryotic and eukaryotic platforms. The ideal platform should consider the natural conformation of the peptide to assure its functional activity, have a low risk for cross contamination, produce significant amounts of the peptide and the peptide should be easily extracted in standard solvents. Finally, production costs should be low enough to allow for further development of a commercial product. Prokaryotic platforms have several advantages; however we are opting for eukaryotic ones and more specifically for transgenic plants as biofactories. Concomitantly, more studies simulating the environment of AMP application are needed, as well as a new strategy for AMP application, since these biomolecules behave differently than classical synthetic pesticides.

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